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Malonylated anthocyanidin 3,5-diglucosides in the flowers of the genus *Disa* (Orchidaceae)

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**Keywords:** *Disa* cultivars; Orchidaceae; cyanidin 3,5-diglucoside; pelargonidin 3,5-diglucoside; malonic acid

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1. **Subject and Source**

Recently we have detected the occurrence of pelargonidin and cyanidin in the flowers of *Disa* hybrids (Tatsuzawa et al., 2010a). In the present study, we further investigated the detailed structures of anthocyanins in the red-purple, red, and orange-red flowers of *Disa* cultivars, grown by Hokkaisankyo Co. Ltd (Hokkaido, Japan), and identified them as acylated and non-acylated pelargonidin and cyanidin 3,5-diglucosides. The distribution of these anthocyanins in Orchidaceae was discussed along with the classification by phylogenetic analysis of Orchidaceae. Voucher specimens are deposited at National Museum of Nature and Science (TNS).

2. **Previous work**

There are only two previous reports on flavonoids from the genus *Disa*. Flavon C-glycosides were detected in leaf material of *Disa uniflora* Berg. (Williams, 1979). More recently, we have reported the distribution of cyanidin and pelargonidin as the aglycones of anthocyanins in the flowers of cultivars of the given genus (Tatsuzawa et al., 2010a).

3. **Present study**

3.1. **Isolation and identification of anthocyanins**

By the analysis of HPLC [HPLC was performed on a LC 10A system
(Shimadzu), using a Waters C18 (4.6 \( \phi \) x 250 mm) column at 40°C with a flow rate of 1 ml/min, the eluate was monitored at 530 nm. The eluant was applied to a linear gradient elution for 40 min from 20 to 85 % solvent B (1.5% \( \text{H}_3\text{PO}_4 \), 20% \( \text{HOAc} \), 25% MeCN in \( \text{H}_2\text{O} \)) in solvent A (1.5% \( \text{H}_3\text{PO}_4 \) in \( \text{H}_2\text{O} \)), more than 20 anthocyanin peaks were observed in the extract from the flowers of red cultivar *Disa* Transvaal ‘Dawn Angel’ (Figure 1). Anthocyanins 1 – 3 were easily identified to be cyanidin 3,5-di-glucoside, pelargonidin 3,5-di-glucoside and cyanidin 3-(6-malonyl)-glucoside-5-glucoside (Figure 2) with authentic samples obtained from the pink and purple flowers of *Centaurea cyanus* (Takeda et al., 1988; Goto and Kondo, 1991) by co-TLC, co-HPLC and UV-VIS spectrometry (Tatsuzawa and Shinoda, 2005) (See in Section 3.1.1. – 3.1.3.). Pigment 5 was identified by the analysis of FAB-MS and \(^1\text{H} \) NMR measurement (Section 3.2.). Pigment 4 was identified by the analysis of partial acid hydrolysis of pigment 5 and FAB-MS (Section 3.3.). Moreover, pigment 6 was identified by the analysis of FAB-MS (Section 3.4.).

Dried corolla mixture of *Disa* red cultivars (60 g) were immersed in 5% HOAc-MeOH (acetic acid-methanol, 5:95, v/v, 500 ml), kept at 4°C for 1 h and extracted. The extract was concentrated to 50 ml. Anthocyanin pigments in the concentrated extract were purified by prep. HPLC [HPLC was performed on a LC 10A system (Shimadzu), using a Waters C18 (19 \( \phi \) x 150 mm) column at 40°C with a flow rate of 1 ml/min, the eluate was monitored at 530 nm.
The eluant was applied to a linear gradient elution for 40 min from 20 to 
85 % solvent B in solvent A| after thin layer and paper chromatography 
(BAW: BuOH-HOAc·H₂O, 4:1:2, v/v/v and 15% HOAc). Finally, pigments 1 
(ca. 0.5 mg), 2 (ca. 0.5 mg), 3 (ca. 0.5 mg), 4 (ca. 3 mg), 5 (ca. 5 mg) and 6 (ca. 2 
mg) were obtained as the major anthocyanins.

On hydrolysis of pigments 4 and 5 with 2N HCl for 3 days at 25°C, 
cyanidin 3,5-diglucoside was obtained in its hydrolysate. Similar hydrolysis 
of pigment 6 afforded pelargonidin 3,5-diglucoside. Moreover, malonic acid 
was detected in both of the hydrolysates.

3.1.1. **Cyanidin 3,5-diglucoside (1)**: UV-VIS in 0.1% HCl-MeOH: \( \lambda_{\text{max}} \)
526,270 nm, \( E_{440}/E_{\text{max}}(\%)=16 \), AlCl₃ shift +, TLC; \( R_f \)-values BAW 
(\( n \)-BuOH-HOAc·H₂O, 4:1:2, v/v/v) 0.02, BuHCl (\( n \)-BuOH-2N HCl, 1:1, v/v, 
upper phase) 0.01, 1%HCl 0.05, AHW (HOAc·HCl·H₂O, 15:3:82, v/v/v) 0.19, 
HPLC; \( t_R \)(min) 13.0.

3.1.2 **Pelargonidin 3,5-diglucoside (2)**: UV-VIS in 0.1% HCl-MeOH: \( \lambda_{\text{max}} \)
507,267 nm, \( E_{440}/E_{\text{max}}(\%)=21 \), AlCl₃ shift 0, TLC; \( R_f \)-values BAW 0.07, 
BuHCl 0.04, 1%HCl 0.13, AHW 0.35, HPLC; \( t_R \)(min) 14.9.

3.1.3. **Cyanidin 3-(6-malonyl)-glucoside-5-glucoside (3)**: UV-VIS in 0.1% 
HCl-MeOH; \( \lambda_{\text{max}} \) 526,278 nm, \( E_{440}/E_{\text{max}}(\%)=16 \), AlCl₃ shift +, TLC;
$R_f$-values BAW 0.08, BuHCl 0.08, 1%HCl 0.04, AHW 0.12, HPLC; $t_R$(min) 16.6.

### 3.2. Pigment 5

The molecular ion [M]$^+$ of pigment 5 was observed at 783 $m/z$ by the FAB-mass analysis, indicating the presence of one molecule of cyanidin and two molecules each of malonic acid and glucose. The FAB-MS ion fragmentation was observed as follows, at $m/z$ 697 [M-86]$^+$ loss of malonic acid; $m/z$ 611 [M-86-86]$^+$ loss of two malonic acids; $m/z$ 535 [M-86-162]$^+$ loss of malonic acid and glucose; $m/z$ 449 [M-86-86-162]$^+$ loss of two malonic acids and one glucose; $m/z$ 287 [M-86-86-162-162]$^+$ loss of two malonic acid and two glucose (=aglycone of cyanidin) supporting that the two malonic acids were linked on cyanidin 3,5-diglucoside. Therefore, the pigment was identified as dimalonyl cyanidin 3,5-diglucoside. The structure of pigment 5 was further elucidated by investigation of its $^1$H NMR spectra [500 MHz for $^1$H spectra in TFA-DMSO-$d_6$ (1:9)], including 2D COSY and negative difference NOE (DIFNOE) spectra. The $^1$H NMR spectrum of 5 showed the presence of one molecule of cyanidin, two molecules each of glucose and malonic acid (see section 3.2.1.). These proton signals were mainly assigned by $^1$H-$^1$H COSY, and linkages between cyanidin and sugars were confirmed by DIFNOE spectra. The proton signals of the sugar parts were observed in the region of $\delta$ 5.54 – 3.21, and two anomic protons were exhibited at $\delta$ 5.54
protons having the coupling constants at $J=7.7$ – 12.2 Hz indicated both glucose units must be $\beta$-glucopyranose. Four methylene protons were assigned to H-6a and 6b of Glc A ($\delta$4.23 and 4.40) and those of Glc B ($\delta$4.14 and 4.48) by the DIFNOE experiments and also were correlated to each anomeric protons by analysis of the $^1$H–$^1$H COSY spectrum. This result indicated that these two glucose units were acylated at the OH-6 groups with acids, respectively. Thus, malonic acids were attached to the OH-6 groups of Glc A and B, respectively.

In order to determined the linkages and position of the glucose units DIFNOE spectra of 5 were measured. Observed NOEs between H-1 of Glc A and H-4 of cyanidin indicates that Glc A is attached to the OH-3 of cyanidin through a glucosidic bond. Glc B was determined to be attached to the OH-5 of cyanidin through a glucosidic bond, because of the presence of NOEs between H-6 of cyanidin and H-1 of Blc B. Therefore, 5 is determined to be cyanidin 3,5-di-O-[6-O-(malonyl)-$\beta$-glucopyranoside] (Figure 2). This is the first report on the presence of cyanidin 3,5-dimalonylglucoside in the Orchidaceae, although this pigment has been found in Lamiaceae plants (Saito and Harborne, 1992) and Dahlia variabilis, belonging to Compositae (Takeda et al., 1986).

3.2.1. Cyanidin 3,5-di-O-[6-O-(malonyl)-$\beta$-glucopyranoside] (5) UV-VIS
in 0.1% HCl-MeOH; $\lambda_{\text{max}}$ 527,278 nm, $E_{440}/E_{\text{max}}$(%)=15, AlCl$_3$ shift +,
TLC; $R_t$-values BAW 0.05, BuHCl 0.07, 1%HCl 0.15, AHW 0.41, HPLC;
$t_R$(min) 20.5, $^1$H NMR: $\delta$ cyanidin: 8.47 (s, H-4), 6.92 (d, J=2.0 Hz, H-6),
6.95 (d, J=2.0 Hz, H-8), 8.04 (d, J=2.4 Hz, H-2'), 7.08 (d, J=8.6 Hz, H-5'),
8.27 (dd, J=2.4, 8.6 Hz, H-6'). Glucose A: 5.54 (d, J=8.0 Hz, H-1), 3.62 (t,
J=8.4 Hz, H-2), 3.45 (m, H-3), 3.28 (m, H-4), 3.95 (m, H-5), 4.23 (m, H-6a),
4.40 (brd, J=12.2 Hz, H-6b). Glucose B: 5.21 (d, J=7.7 Hz, H-1), 3.51 (m,
H-2), 3.21 (m, H-3), 3.25 (m, H-4), 3.82 (m, H-5), 4.14 (dd, J=7.7, 11.9 Hz,
H-6a), 4.48 (m, H-6b). Malonic acid (attached to OH-6 of Glc A): $-\text{CH}_2\cdot$
3.44 (s). Malonic acid (attached to OH-6 of Glc B): $-\text{CH}_2\cdot$: 3.43 (s).

3.3. Pigment 4

The molecular ion [M]$^+$ of pigment 4 was observed at 697 $m/z$ by the
FAB-mass analysis indicating the presence of one molecule of cyanidin and
malonic acid, and two molecules of glucose. The FAB-MS fragmentation at
611 $m/z$ [M-86]$^+$ loss of malonic acid, at 449 $m/z$ [M-86-162]$^+$ loss of malonic
acid and glucose, and at 287 $m/z$ aglycone indicated that the malonic acid
was linked one of the glucoses of cyanidin 3,5-diglucoside. In order to obtain
the authentic anthocyanin, cyanidin 3-glucoside-5-(6-malonylglucoside, the
partial acid hydrolysis of pigment 5 was performed by the procedure
described previously (Saito et al., 2008) providing cyanidin 3,5-diglucoside (=
pigment 1), cyanidin 3-(6-malonylglucoside)-5-glucoside (= pigment 3), and
cyanidin 3-glucoside-5-(6-malonylglucoside) as the major anthocyanin products from the hydrolysate. The structures of these pigments were confirmed by the analysis of TLC, HPLC and FAB mass spectra. By direct comparison of pigment 4 with one of the partial hydrolysate of pigment 5, cyanidin 3-glucoside-5-(6-malonylglucoside), both pigments were identical to by the analysis of TLC, HPLC, and the properties of UV and Vis. Therefore, pigment 4 is cyanidin 3-O-glucoside-5-O-(6-O-malonyl)-glucoside (Figure 2), which is a new anthocyanin in plant (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Honda and Saito, 2002).

3.3.1. Cyanidin 3-glucoside-5-(6-malonyl)-glucoside (4)  UV-VIS in 0.1% HCl-MeOH; $\lambda_{\text{max}}$ 526,278 nm, $E_{440}/E_{\text{max}}(\%)=16$, AlCl$_3$ shift +, TLC; $R_f$-values BAW 0.06, BuHCl 0.06, 1%HCl 0.05, AHW 0.16, HPLC; $t_R$(min) 18.0.

3.4. Pigment 6 and non purified pigments A and B

The molecular ion [M]$^+$ of pigment 6 was observed at 767 $m/z$ by the FAB-mass analysis indicating the presence of one molecule of pelargonidin and two molecules each of malonic acid and glucose. The FAB-MS fragmentations at 681 $m/z$ [M-86]$^+$ loss of malonic acid, at 519 $m/z$ [M-86-162]$^+$ loss of malonic acid and glucose, at 271 $m/z$ aglycone suggesting that the two malonic acids were linked on pelargonidin 3,5-diglucoside.
Therefore, the pigment 6 was identified as dimalonyl pelargonidin 3,5-diglucoside. By the partial hydrolysis of pigment 6 with 2N HCl for 24 h at 25°C, pelargonidin 3,5-diglucoside (pigment 2), pigment A \[t_R \text{(min)} 18.7\] and pigment B \[t_R \text{(min)} 19.9\] were detected by the analysis of HPLC. Therefore, pigments 2, A, B and 6 were deduced as the pelargonidin derivatives as for the cyanidin derivatives of pigments 1, 3, 4 and 5, respectively. Further structure elucidation of these pigments could not be carried out because of small amounts available. Therefore, these three anthocyanins were tentatively determined to be pelargonidin 3-(6-malonyl)-glucoside-5-glucoside as pigment A, pelargonidin 3-glucoside-5-(6-malonyl)-glucoside as pigment B and pelargonidin 3,5-di-(6-malonyl)-glucoside as pigment 6, respectively, at present.

3.4.1. Dimalonyl pelargonidin 3,5-diglucoside (6): UV-VIS in 0.1% HCl-MeOH: \(\lambda_{\text{max}}\) 510,268 nm, \(E_{440}/E_{\text{max}}\)(%)=20, AlCl_3 shift 0, TLC; \(R_f\) values BAW 0.15, BuHCl 0.14, 1%HCl 0.24, AHW 0.54, HPLC; \(t_R\)(min) 22.3.

3.5. Distribution of anthocyanins

Dried Disa flowers of ca. 10 mg each in dry weight of five cultivars were immersed in MAW (MeOH-HOAc-H_2O, 4:1:5, v/v/v, 1ml) and extracted. Analytical HPLC was performed on a LC 10A system (Shimadzu), using a
Waters C18 (4.6 φ x 250 mm) column at 40°C with a flow rate of 1 ml/min, the eluate was monitored at 530 nm. The eluant was applied to a linear gradient elution for 40 min from 20 to 85 % solvent B in solvent A. The results of HPLC measurement at 530 nm are as follows:

3.5.1. *Disa* Child Safety Transvaal ‘Dawn Angel’: 1 (6.3%), 2 (2.6%), 3 (9.0%), 4 (31.6%), 5 (37.8%), 6 (2.4%), A (1.1%) and B (3.3%).

3.5.2. *D. Foam* ‘San Francisco’: 1 (4.4%), 2 (1.4%), 3 (7.1%), 4 (29.2%), 5 (38.3%), 6 (9.8%), A (2.2%) and B (5.2%).

3.5.3. *D. Santa Rosa* ‘Purple Taffy’: 1 (7.9%), 2 (0.9%), 3 (2.4%), 4 (32.5%), 5 (18.1%), 6 (1.8%), A (0.1%) and B (1.0%).

3.5.4. *D. Sid Cywes* ‘Marlene’: 1 (3.0%), 2 (2.1%), 3 (5.0%), 4 (30.6%), 5 (37.1%), 6 (11.3%), A (1.2%) and B (6.0%).

3.5.5. *D. Unilangley* ‘Pink Tourmaline’: 1 (5.9%), 2 (1.2%), 3 (5.7%), 4 (53.8%), 5 (26.2%), 6 (0.3%), A (0.1%) and B (1.8%).

4. Chemotaxonomic significance

The occurrence of pelargonidin glycosides in the flowers of orchids were previously established with thin layer and paper chromatography using crude extracted pigments of the genera *Brassotonia, Broughtonia, Cattleopsis* and *Cattleytonia* (Arditti, 1969). However, the identification procedures of these orchid anthocyanins are considered to be rather out of
date and also lacking reliability due to absence of the data of the analysis by HPLC, MS and so on (Griesbach, 1990). Therefore, the present results were the exact report in which the distribution of pelargonidin glycosides and acylated pelargonidin glycosides are confirmed in orchids.

Recently, anthocyanins have been used in chemotaxonomic studies of Orchidaceae (Strack et al., 1986, 1989; Saito et al., 1994, 1995; Williams et al., 2002; Fossen and Øvstedal, 2003; Tatsuzawa et al., 1994, 1996a,b, 1997, 1998, 2004, 2005, 2006, 2010b). These studies included the genera Anacamptis, Barlia, Bletilla, Cattleya, Cephalanthera, Cymbidium, Dactylorhiza, Dendrobium, Dracula, Epipactis, Gymnadenia, Himantoglossum, Laelia, xLaeliocattleya, Limodorum, Neottianthe, Nigritella, Ophrys, Orchis, Phalaenopsis, Serapias, Sophronitis, Traunsteinera and Vanda. Among these genera, the 3,5-diglucoside pattern of anthocyanidin glycosides (including 3-glucoside and 3,7-diglucoside patterns) were detected from Anacamptis, Barlia, Cephalanthera, Dactylorhiza, Epipactis, Gymnadenia, Himantoglossum, Limodorum, Neottianthe, Nigritella, Ophrys, Orchis, Serapias and Traunsteinera as their main anthocyanins (Strack et al., 1989). From a standpoint of the phylogenetic classification of Orchidaceae, the genera Cephalanthera, Epipactis and Limodorum belong to subfamily Epidendroideae tribe Neottieae (Pridgeon et al., 2005), and the others belong to subfamily Orchidoideae tribe Orchideae (Pridgeon et al., 2001). In this study we found another member of the 3,5-diglucoside pattern of
anthocyanins for *Disa*. As the genus of *Disa* belongs to subfamily Orchidoideae tribe Diseae (Pridgeon et al., 2001), Diseae is the third orchid tribe other than Neottieae and Orchideae in which the 3,5-diglucoside pattern of anthocyanins were found.

To date, the distribution of 3,5-di-malonylglucosilated anthocyanins has been reported in the two families, Compositae and Labiatae (Takeda et al., 1986; Saito and Harborne, 1992). From a chemotaxonomical point of view, since *Disa* anthocyanins 5 and 6 were found to be 3,5-di-malonylglucosilated anthocyanins, the family of Orchidaceae, to which *Disa* belongs, should to be added to the above two families.

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February 7, 2011

Dr. M.S.J. Simmonds
Editor-in-Chief of Biochemical Systematics and Ecology

Dear Dr. M.S.J. Simmonds

Thank you very much for your e-mail dated February 4, 2011. I am sending the revised manuscript entitled “Malonylated anthocyanidin 3,5-diglucosides in the flowers of the genus Disa (Orchidaceae)” by Fumi Tatsuzawa, Kazumitsu Miyoshi, Tomohisa Yukawa, Koich Shinoda, Kenjiro Toki, Norio Saito, Atsushi Shigihara, Toshio Honda.

We have revised all the points suggested by you as follows.

(1) According to the reviewer’s comment, we mentioned Williams’ work in the text and quoted as the reference.
(2) Although the NMR data for compounds 4 and 6 were not measured, unfortunately, the structure of 4 was unambiguously determined by direct comparison with the authentic specimen obtained by the hydrolysis of 5. Therefore, we rewrote the sentence about the structures for pigments 4-6 in section 3.1.
(3) Section 3.2, p.5: We changed ‘malonic acid was’ to ‘malonic acids were’.
(4) Section 3.2, p.5: We changed ‘pigments 5’ to ‘pigment 5’.
(5) Section 3.2, p.6: According to the comment, the sentence ‘Moreover, irradiations at H-1 of Glc A and B …… were observed.’ was deleted from section 3.2. Moreover, the sentence ‘Thus, malonic acids were attached to the OH-6 group of Glc A and B, respectively.’ was moved after the sentence ‘This result indicated that those two glucose units were acylated at the OH-6 groups with acids, respectively.’
(6) Section 3.2.1, p.7: According to the comment, we changed the coupling constants of H-5s, from ‘t, ……’ to ‘m’.
(7) Section 3.4, p.8: We changed ‘acid was’ to ‘acids were’.
(8) Section 4, p.10: According to the comment, we changed ‘detected’ to ‘established’.
(9) Section 4, p.11: We changed ‘This is’ to ‘Therefore, the present results were’.
(10) Section 4: According to the comment, the sentence ‘To date, the distribution of 3,5-di-malonylglucosilated anthocyanins has been reported in the two families, Compositae and Labiatae (Takeda et al., 1986; Saito and Harborne, 1992). From a
chemotaxonomical point of view, since *Disa* anthocyanins 5 and 6 were found to be 3,5-di-malonylglucosilated anthocyanins, the family of Orchidaceae, to which *Disa* belongs, should to be added to the above two families.’ was added to the section 4.

(11) References: We changed ‘Europian’ to ‘European’.

I hope that our manuscript will now be deemed worthy of publication in Biochemical Systematics and Ecology. Again, we thank you for your consideration of this manuscript.

With best regards,

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Figure 1. HPLC profile for anthocyanins (530 nm) in the red flower extract of *Disa* Sid Cywes ‘Marlene’.

Pigments 1 - 6 are purified. Pigments A and B are not purified.
Figure 2. Anthocyanins from *Disa* cultivars.

1: R=OH, 2: R=H, 3: R=OH, 4: R=OH, 5: R=OH
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Anthocyanins from *Disa* cultivars.
1: R=OH, 2: R=H, 3: R=OH, 4: R=OH, 5: R=OH